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TITLE: Preclinical Testing the Therapeutic Potential of a Potent and Novel Small-molecule Inhibitor of Bcl-2 as a Novel Therapy for Hormone-refractory Prostate Cancer

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14. ABSTRACT Targeting the anti-apoptotic Bcl-2 members using non-peptide, small-molecule inhibitors is a new and exciting therapeutic strategy. Our work has led to the discovery of potent, non-peptide small-molecule inhibitor apogossypolone that not only binds to Bcl-2 and Bcl-xL proteins but also Mcl-1. Consistent with its strong binding affinity to Bcl-2 members, apogossypolone potently and effectively inhibits cancer cell growth in androgen-independent human prostate cancer PC-3 and DU-145 cell lines. Apogossypolone is well-tolerated in animals and has an excellent oral bioavailability. Our studies using the PC-3 androgen-independent xenograft model showed that Apogossypolone can enhance the antitumor activity of taxotere without causing any additional signs of toxicity, as compared to taxotere alone. Apogossypolone may have a promising therapeutic potential to be developed as an effective and non-toxic new therapy for the treatment of advanced, androgen-independent human prostate cancer.

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Table of Contents

Introduction: Current chemotherapy has limited effectiveness in hormone-refractory prostate cancer and new therapeutic strategies are urgently needed to improving the survival and quality of life in patients with hormone-refractory prostate cancer.

Current chemotherapeutic agents most commonly work by directly or indirectly inducing apoptosis, or programmed cell death in tumor cells. The impaired ability of prostate cancer cells to undergo apoptosis plays a key role in the resistance of prostate cancer cells to chemotherapy or radiation and for the failure of current treatment protocols for hormone-refractory prostate cancer. Hence, current and future efforts for designing new therapies to treat hormone-refractory prostate cancer must include strategies that specifically target resistance of prostate cancer cells to apoptosis.

Bcl-2 is a potent cellular inhibitor of apoptosis. Bcl-2 is overexpressed in 30-60% of prostate cancer at diagnosis but in nearly 100% of hormone-refractory prostate cancer. Prostate cancers that express high level of Bcl-2 are often resistant to chemotherapeutic agents or radiation therapy. Therefore, overexpression of Bcl-2 may play an important role to the high failure rate for current treatment of hormone-refractory prostate cancer. Hence, inhibition of the anti-apoptotic function of Bcl-2 represents a promising strategy for overcoming the resistance of prostate cancer to chemotherapy or radiation therapy and for developing an entirely new class of anticancer drugs for treatment of prostate cancer, especially hormone-refractory prostate cancer.

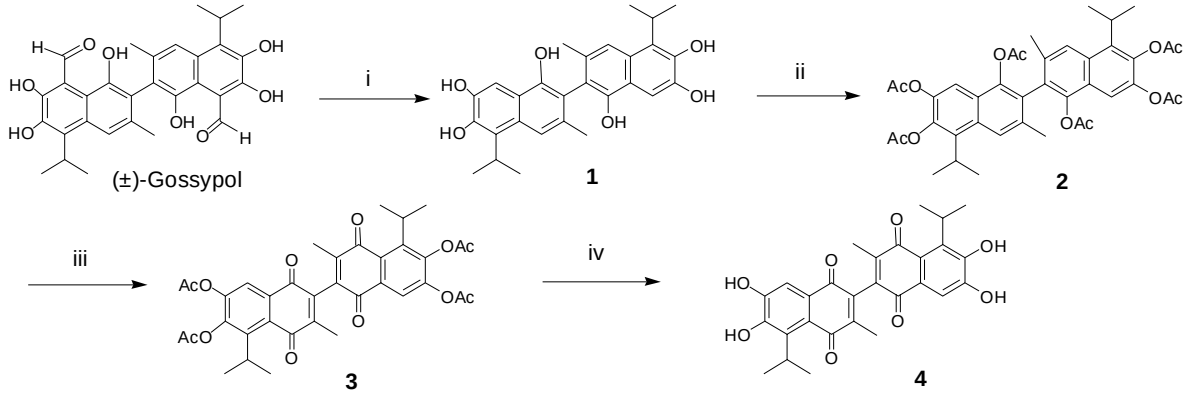
In this idea grant, we have proposed to test a potent and novel small-molecule inhibitor that we have discovered and synthesized in our laboratory for its mechanism of action and therapeutic potential for the treatment of human prostate cancer.

Body of the report:

Task 1. Development of an efficient synthetic procedure for the synthesis of our target compound, apogossypolone.

We have developed an efficient synthetic method for apogossypolone, as shown in **Scheme I**. Racemic gossypol was treated with 40 % sodium hydroxide at 85 °C for 2 h to remove the aldehyde groups, followed by acetylation using acetic anhydride to afford the (±)-hexaacetylapogossypol **2**. The yield for the two steps was 82 %. Compound **2** was transferred to (±)-tetraacetylapogossypolone **3** by periodic oxidation in 55 % yield. Deprotection of **3** in the presence of potassium carbonate in dioxane afforded (±)-apogossypolone **4** in 98 % yield. We have synthesized grams of apogossypolone using this method.

Scheme I. Synthesis of apogossypolone.



Reagents and conditions: (i) 40 % NaOH, 85 °C, 2 h; (ii) Ac_2O, iPr_2NEt, CH_2Cl_2, room temperature, 12 h, 82 % for two steps; (iii) Periodic acid, dioxane, 95 °C, 15 min, 55 %; (iv) K_2CO_3, dioxane, 70 °C, 5 h, then 4 M HCl, 98 %

We further determined that in contrast to its parent compound gossypol, which has stable (+)- and (-)-gossypol (two enantiomers), apogossypolone has a single stable isomer. **Task #1 has been successfully completed**.

Task 2. Evaluation of the binding affinities of apogossypolone to anti-apoptotic Bcl-2 proteins

We have developed sensitive and robust binding assays for the Bcl-2 and Bcl-xL proteins and evaluated the binding affinities of apogossypolone to both Bcl-2 and Bcl-xL. During the course of this project, we have also developed a sensitive, fluorescence-polarization assay for another Bcl-2 member, Mcl-1. In the last several years, Mcl-1 has emerged as an important molecular target and displays a different binding specificity for other pro-apoptotic Bcl-2 members. For example, Bcl-2 and Bcl-xL bind to Bid and Bim and Bad with high affinities but don't bind to Noxa. In comparison, Mcl-1 binds to Bid, Bim and Noxa with high affinities but does not bind to Bad. Potent and specific small-molecule inhibitors of Bcl-2 and Bcl-xL have no or minimal activity in cancer cells with overexpression of Mcl-1 but become highly effective when Mcl-1 is neutralized.

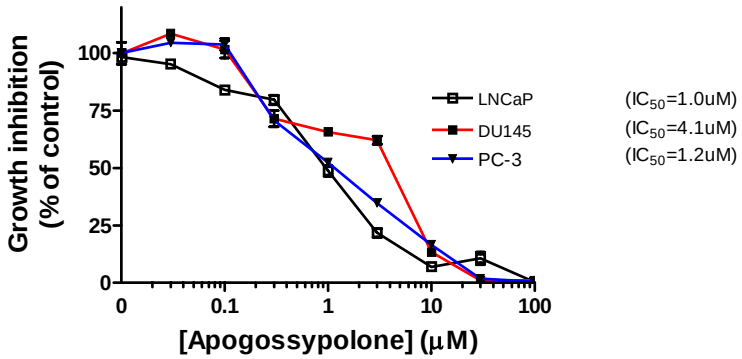
We have determined the binding affinities of apogossypolone to Bcl-2, Bcl-xL and Mcl-1 proteins using these sensitive and quantitative assays and shown that apogossypolone binds to Bcl-2, Bcl-xL and Mcl-1 proteins with Ki values of 170, 660 and 51 nM, respectively. Therefore, apogossypolone is a potent small-molecule inhibitor against multiple Bcl-2 proteins. Recently studies on inhibitors that specifically target Bcl-2 and Bcl-xL show that such inhibitors (e.g. ABT-737 developed from Abbott's Laboratories) are only active in a small subset of human cancer cell lines with low levels of Mcl-1 protein. Down-regulation of Mcl-1 can dramatically sensitize cancer cells to ABT-737. Hence, apogossypolone, which targets not only Bcl-2 and Bcl-xL proteins, but also Mcl-1, may have a major advantage over inhibitors that only target Bcl-2 and Bcl-xL and spare Mcl-1 protein. **Task #2 has been successfully completed.**

Task 3. Evaluation of the activity of apogossypolone in human prostate cancer cell lines

We have first evaluated apogossypolone for its ability to inhibit cancer cell growth in three different prostate cancer cell lines. The results are shown in **Figure 1**. Our data showed that apogossypolone not only potently inhibits cell growth in the LNCaP androgen-sensitive human prostate cancer cell line with an IC_{50} value of 1.0 μM, it also potently inhibits the growth of androgen-independent human prostate cancer cell lines.

The IC_{50} values obtained using a 4-day MTT cell growth assay are 1.2 and 4.1 μM, respectively, in the PC-3 and DU-145 androgen-independent prostate cancer cell lines. Hence, apogossypolone is effective in inhibition of cell growth against androgen-independent prostate cancer cell lines.

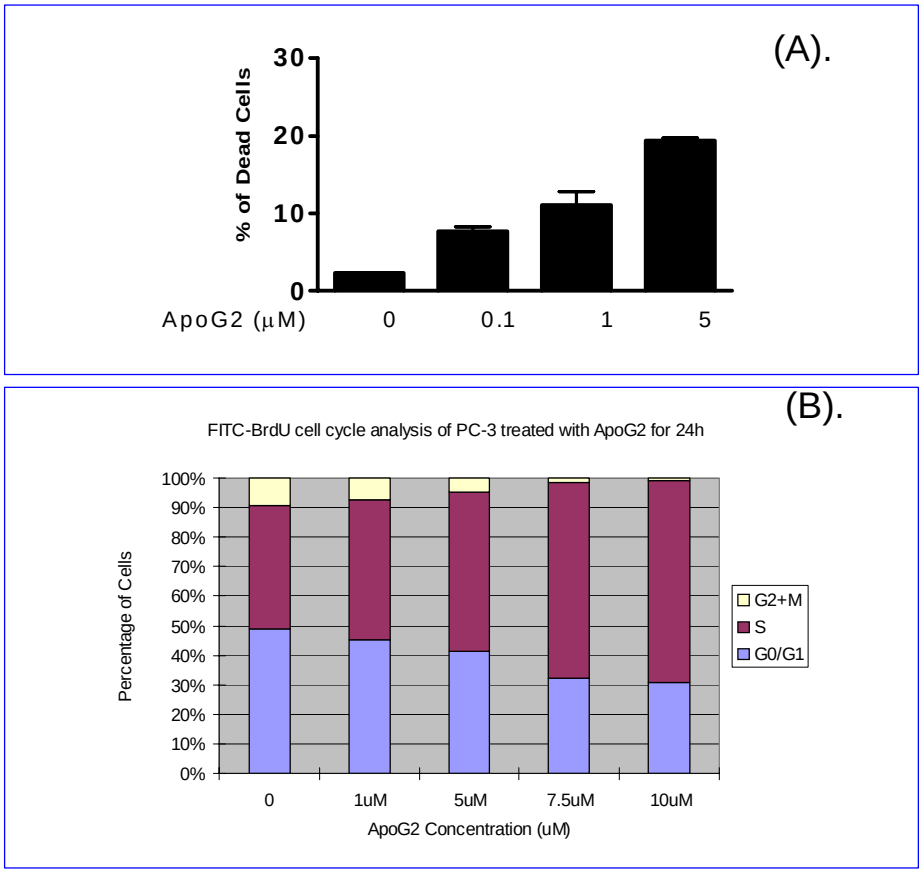
Figure 1. Inhibition of cell growth by apogossypolone in three prostate cancer cell lines.



We next performed analysis on Apogossypolone in the PC-3 cancer cell line and the results are summarized in **Figure 2.**

Our data showed that Apogossypolone (ApoG2) can induce significant cell death in PC-3 cancer cells (Figure 2A) at as low as 100 nM concentration, consistent with its strong binding affinity to Bcl-2/Bcl-xL/Mcl-1 proteins. Very interestingly, our cell cycle analysis showed that Apog2 has a fairly strong cell cycle effect (Figure 1B) and causes blockage from G1 phase transition to G2/M phase. Therefore, the sactivity by ApoG2 in cell growth inhibition in PC-3 cells is a combination of cell death induction and cell cycle arrest. **Task #3 has been successfully completed.**

Figure 2. (A). Induction of cell death in PC-3 cells by Apogossypolone (ApoG2). Cells were treated by ApoG2 for 4 days and cell viability was determined using trypan blue exclusion assay. (B). Cell cycle analysis. Cells were treated by ApoG2 for 24 hours and cell cycle was performed by flow cytomertric analysis using FITC-BrdU labeling.



Task 4: Evaluations of the *in vivo* toxicity and pharmacokinetics of apogossyplone in animals

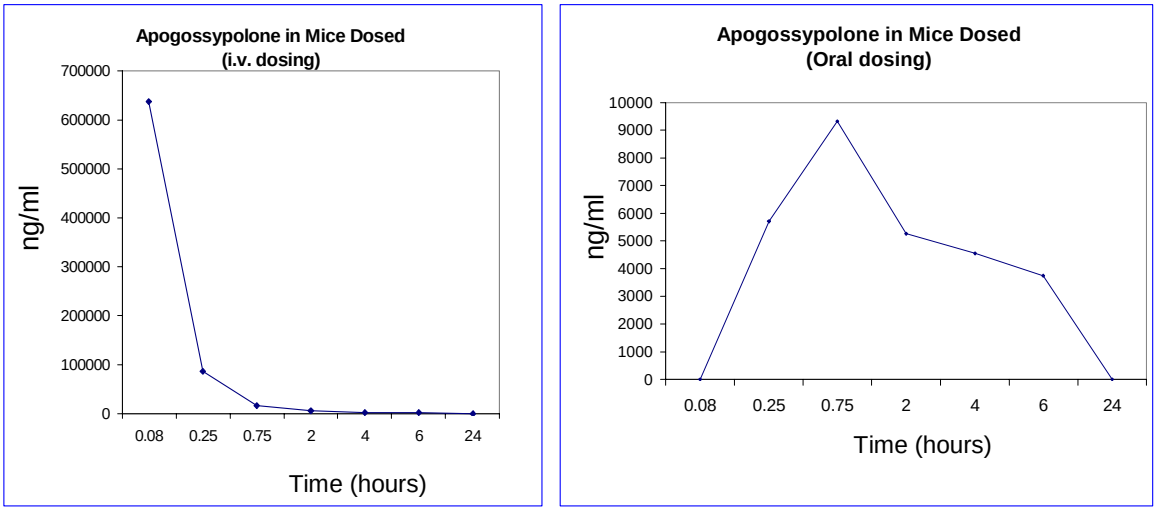
We have tested apogossypolone extensively in mice for its maximum tolerated dose (MTD). It was found that apogossypolone was well tolerated. Mice dosed at 80 mg/kg intravenously daily for 2 weeks showed no weight loss or other signs of toxicity. Furthermore, mice dosed at 240 mg/kg *via* oral gavage (oral dosing) daily for 2 weeks also did not show weight loss or other signs of toxicity. In fact, apogossypolone at 400 mg/kg via oral gavage (oral dosing) daily for 2 weeks is well tolerated in mice.

Hence, in direct comparison to gossypol, apogossypolone is at least >10 times more tolerated in mice. Our data thus indicated that apogossypolone is well tolerated in mice and has a much reduced toxicity as compared to its parent compound gossypol, consistent with our initial prediction that removal the two aldehyde groups in gossypol would greatly reduce the toxicity of this compound to animals.

We next determined the pharmacokinetics (PK) of apogossypolone to assess its bioavailability. Since it is highly desirable to develop an orally available anticancer drug, we have performed our PK studies using both i.v. and oral routes of administration of the drug. The data are summarized in **Figure 3**.

As can be seen, apogossypolone has a good PK profile in oral dosing. At 30 mg/kg, apogossypolone has a cMax of 9000 ng/ml (18 μM since the molecular weight of apogossypolone is 492.5). Although its $T_{1/2}$ is only 1 hour in oral dosing, very high concentration of the drug was observed 6 hours after the dosing with a concentration of 3500 ng/ml (7 μM). Taken together, our toxicity and pharmacokinetic studies demonstrate that apogossypolone is well-tolerated in mice and has a good pharmacokinetic profile when given orally. **Task #4 has been successfully completed.**

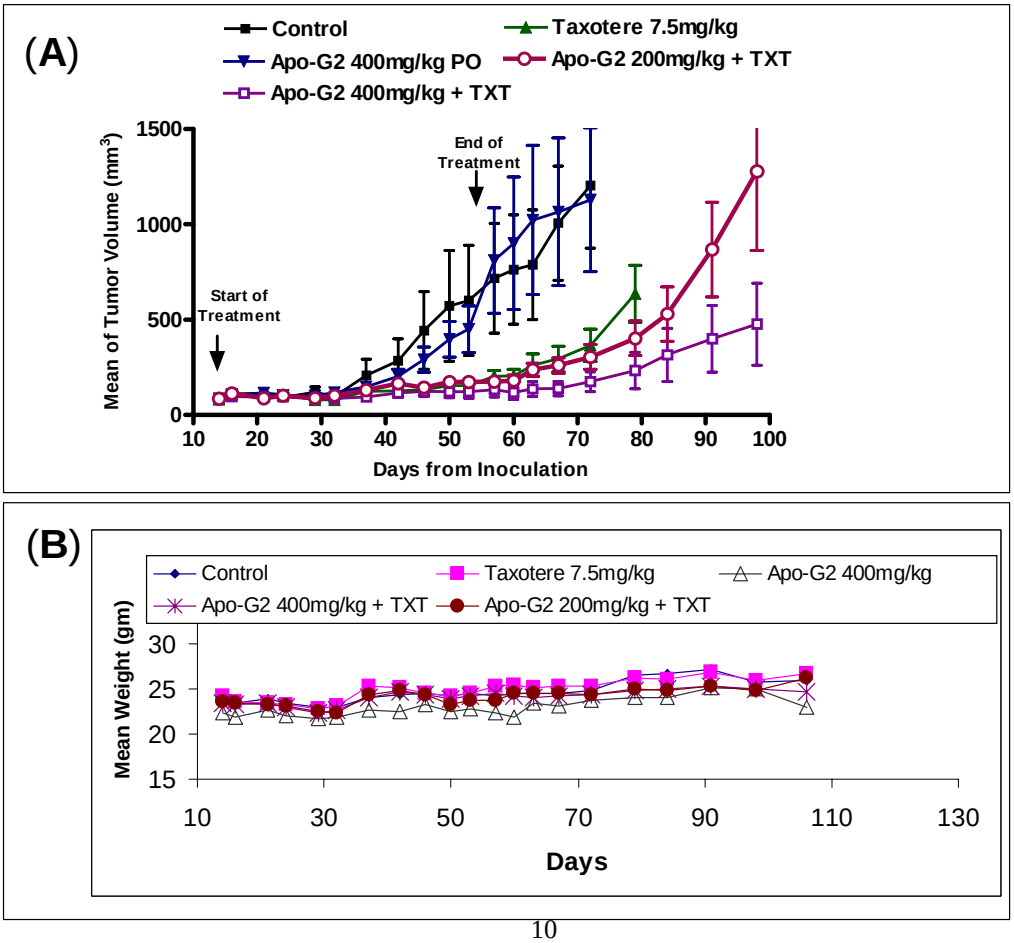
Figure 3. Determination of the pharmacokinetics of apogossypolone in mice. A group of mice were dosed with a single dose of apogossypolone at 30 mg/kg and plasma were collected at 0 min, 5, 15, 45 min, and 2, 4, 6 and 24 hours. Each group consisted of 2 mice. Samples were analyzed using a highly sensitive LC-MS-MS method developed for apogossypolone.



Task 5. *in vivo* antitumor activity of Apogossypolone alone and in combination with other chemotherapeutic agents

We next evaluated the antitumor activity of apogossypolone alone and in combination with other chemotherapeutic agents. We have employed the PC-3 androgen-independent xenograft model based upon our *in vitro* data. In addition to test apogossypolone as a single agent, we also tested its combination with taxotere, a clinically approved agent for the treatment of advanced, androgen-independent prostate cancer. The results are summary in **Figure 4**.

Figure 4. (A). Antitumor activity of Apogossypolone (Apo-G2) alone and in combination with taxotere. Animals bearing PC-3 xenograft Tumors were treated when tumors reached an average size of 80 mm^3. Each treated group consisted of 7-8 tumors/animals and the control group had 10 tumors/animals. Apogossypolone was given daily, *via* oral gavage, for a total of 4 weeks. Taxotere was given i.v. weekly for 3 weeks. (B). Mean of animal weight.



Despite its potent *in vitro* activity and excellent pharmacokinetic profile, apogossypolone by itself has no significant *in vivo* activity when given orally in inhibition of tumor growth in the PC-3 xenograft model. Taxotere, a clinically proved agent used for the treatment of androgen-independent prostate cancer, is effective in inhibition of tumor growth as a single agent. Despite the lack of *in vivo* activity as a single agent, the combination of apogossypolone with taxotere is more effective than taxotere alone. While it took approximately 80 days for tumors to reach a mean volume of 500 mm^3 in the taxotere-treated group, greater than 98 days was needed for the tumors to reach the same volume in the group of animals treated by the combination of apogossypolone with taxotere. Both treated groups achieve high statistical significance as compared to the untreated control ($p < 0.05$). Of note, the animals treated with taxotere alone, apogossypolone alone or the combination did not experience significant weight loss during and after the treatment.

Taken together, our *in vivo* studies provide evidence that apogossypolone in combination with taxotere should be further evaluated as a potentially promising new protocol for the treatment of androgen-independent human prostate cancer. **Task #5 has been successfully completed.**

Key Research Accomplishments

(1). We have developed an efficient synthetic method to synthesize apogossypolone. This method is suitable for synthesis of large quantity of apogossypolone (>100 grams).

(2). We have developed several biochemical assays to assess the binding affinities of apogossypolone to multiple anti-apoptotic Bcl-2 proteins, including Bcl-2, Bcl-xL and Mcl-1. Using these methods, we showed that apogossypolone binds to Bcl-2, Bcl-xL and Mcl-1 with high affinities. The ability of apogossypolone to target more than anti-apoptotic Bcl-2 proteins may prove to be an advantage over other small-molecule inhibitors that only target Bcl-2/xL or Mcl-1 protein.

(3). Using multiple assays, we have shown that apogossypolone is effective in inhibition of cell growth, induction of apoptosis and cell cycle arrest in androgen-independent human prostate cancer models.

(4). We have shown that apogossypolone is extremely well-tolerated in animals and is at least 10-times more tolerated than its parent analogue gossypol, consistent with our design rationale and initial prediction.

(5). We have determined that apogossypolone has an excellent pharmacokinetic profile and is orally bioavailable, a major advantage for the development of a new anticancer agent.

(6). We showed that although apogossypolone on its own has a minimal antitumor activity in an animal model of human androgen-independent prostate cancer, the combination of apogossypolone with taxotere is more effective than taxotere alone. Furthermore, the combination of apogossypolone with taxotere displays no signs of toxicity to animals.
Collectively, our study has laid the foundation for the advanced preclinical development of apogossypolone as a new anticancer drug, either alone, or in combination with taxotere.

Reportable Outcomes:

(1). A patent application has been filed on the discovery of apooysspolone and methods of use for the treatment of human prostate and other types of cancer.

(2). A manuscript described the design, synthesis and initial evaluation of apogossypolone as potent small-molecule inhibitors of Bcl-2/Bcl-xL/Mcl-1 has been completed and will be submitted soon. The support from the DOD prostate cancer program is acknowledged.

(3). Second manuscript on detailed *in vitro* and *in vivo* evaluations of apooysspolone in human prostate cancer models will be prepared and submitted. The support from the DOD prostate cancer program is acknowledged.

Conclusions: Targeting the anti-apoptotic Bcl-2 members using non-peptide, small-molecule inhibitors is a new and exciting therapeutic strategy. Our work has led to the discovery of potent, non-peptide small-molecule inhibitor apogossypolone that not only binds to Bcl-2 and Bcl-xL proteins but also Mcl-1. Consistent with its strong binding affinity to Bcl-2 members, apogossypolone potently and effectively inhibits cancer cell growth in androgen-independent human prostate cancer PC-3 and DU-145 cell lines. We demonstrated that apogossypolone effectively induces cell death and also cell cycle arrest. We have determined that apogossypolone is well-tolerated in animals and has an excellent oral bioavailability and pharmacokinetic profiles. *In vivo* studies using an animal model of androgen-independent PC-3 xenograft model showed that while oral administration of apogossypolone has no significant antitumor activity on its own, the combination of apogossypolone with taxotere is more effective than taxotere in inhibition of tumor growth. Importantly, the combination displays no sign of toxicity to animals. Taken together, our study shows that apogossypolone represents a promising, orally available, potent small-molecule inhibitor of Bcl-2/Bcl-xL/Mcl-1 to be developed for the treatment of advanced, androgen-independent human prostate cancer.